General Comments

It was not the purpose of this review to present a comprehensive survey of hapten–protein conjugates, but rather to provide sufficient information to guide the researcher in the design of his or her particular experiments. On the other hand, the most practical approaches to the preparation of hapten–protein conjugates were cited.

Many of the methods used to prepare immunogenic conjugates have also been used to link drugs to carrier molecules (including antibodies) in order to "target" cytotoxic drugs. Two reviews that are useful in that they describe many of the methods used to make the carrier–drug conjugates are those by Trouet[131] and by Ghose.[132] The information in these reviews should be useful to immunologists as well.

[131] A. Trouet, *Eur. J. Cancer* **14**, 105 (1978).
[132] T. Ghose, *J. Natl. Cancer Inst.* **61**, 657 (1978).

[5] Production of Reagent Antibodies

By B. A. L. Hurn and Shireen M. Chantler

Immunization

The explosion of interest in immunoassay procedures during the last two decades has resulted in an enormous volume of literature describing, for the most part, satisfactory results of immunization. During the same period, knowledge of the underlying mechanisms of the immune response has advanced greatly from an original state of almost total ignorance. Perhaps unfortunately, those who have pursued basic understanding have seldom been much concerned with the practical problems of making useful reagent antibodies. As a result, with few exceptions the literature of immunization does no more than describe successful procedures, and the variety of these is legion. In the usual way of things, abortive attempts are seldom mentioned, let alone described, yet anyone with practical experience who has also discussed the matter with colleagues will be well aware that all the successful methods have also, at other times or in other places, singularly failed to give the desired results. Not surprisingly, the failure rate is higher when making antisera for more demanding test systems, such as radioimmunoassay, than for immunoprecipitin methods, for instance. Much of the uncertainty over the outcome of immunization may be ascribed to variations in individual animal response; however, when an

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experimental comparison of different procedures is made in such a way as to overcome the effect of individual animal variation, the results may well be inconclusive or irreproducible despite the considerable effort involved.[1]

Regrettably, then, it must be said that information concerning methods of immunizing laboratory animals is almost entirely anecdotal. The available evidence strongly suggests that there are influences as yet unrecognized that may be as important to success as any of the factors already known. Nevertheless, while acknowledging the significance of art, green fingers, or even plain luck, it is worth considering the known factors briefly so as to provide some evidence in support of the methods of immunization recommended later; they are related to the immunogen, the adjuvant, the choice of animal, the route of injection, and the dosage schedule.

The Immunogen

Particulate (cellular) materials, such as heterologous erythrocytes or bacteria, are usually intensely immunogenic, producing a rapid response when administered without adjuvant of any sort. The major problem likely to be encountered is lack of the desired specificity in the resultant antiserum, since the particles have a complex antigenic structure much of which may be shared with other more or less closely related cell types. Short immunization courses are usually adequate but often give rise to a high proportion of IgM antibody, which may be very satisfactory in agglutination techniques but tends to be less stable during storage than IgG.

Most antigens of interest to immunoassayists are soluble materials that vary greatly in their immunogenicity dependent on their chemical structure and molecular size. Since soluble substances are readily cleared from the circulation, either by some metabolic pathway or by excretion, through routes that largely bypass lymph nodes, spleen, and other reservoirs of immunopotent cells, they rarely stimulate the production of effective reagent antibodies unless administered with some sort of adjuvant, as described below. Even then, they vary widely in immunogenicity.

Proteins and the larger polypeptides of molecular weight greater than about 5000 will readily stimulate a potent immune response. Many may exist in dimer or polymer form, either naturally or as a result of minor denaturation during purification, and this may increase their immunogenicity (major denaturation may be associated with loss of native antigenic characteristics, however, and should be avoided). The smaller the peptide

[1] S. Lader, B. A. L. Hurn, and G. Court, *in* "Radioimmunoassay and Related Procedures in Medicine," p. 31. International Atomic Energy Agency, Vienna, 1974.

within the molecular weight range of 5000–1000, the more difficult it seems to be to make avid antisera, although the correlation is much less than perfect. In this size range, closely related (or even identical) peptides are found in all the usual species of laboratory animal, so the element of "foreigness" of the antigen is lost. Many small peptides may lack the clearly defined tertiary structure that is presumably necessary for a substance to be recognized as a unique antigen. Finally, degradation of these substances in the tissues and circulation, by specific enzymes and by nonspecific proteases, may well be so brisk as to prevent effective contact with immunopotent cells.

With the exception of some of the larger polysaccharide molecules, no substances other than the proteins and larger polypeptides are effective immunogens in themselves. Nevertheless, antisera of high avidity and specificity can be raised to steroids, glycosides, oligopeptides, and the like if they are first chemically bonded to a large carrier molecule, preferably a protein that is in itself immunogenic in the species under immunization. Current immunological theory suggests that the initial stages of immunization require cooperation between T and B lymphocytes, the T lymphocytes first binding with a recognizably "foreign" substance and then presenting the bound antigen to B lymphocytes bearing suitable receptors. This cooperation is impossible if the antigen is too small to be shared between T and B cells, but a complex of the antigen with a suitable carrier becomes fully effective. Small, nonimmunogenic antigens of this type are known as haptens and, in the form of drugs, steroid hormones and small peptides, have been of great interest to immunoassayists during the last decade. The method of coupling carrier and hapten should be carefully chosen so as to avoid unwanted structural alteration of the latter and so that the linkage does not involve the immunochemically distinctive part of the hapten molecule. Antibodies produced in response to immunization with conjugated haptens generally "recognize" that part of the hapten farthest from the point of linkage, which thus determines their specificity. Highly substituted carriers are usually most effective, and molar ratios of 15–30:1 (hapten:carrier) are desirable, when possible. For best results the carrier should be a protein foreign to the immunized species—thyroglobulin and keyhole limpet hemocyanin are used quite widely, but bovine (or other) serum albumin is fully effective and more easily available. The subject has recently been well reviewed in relation to steroid conjugates by Pratt.[2]

The purity of the immunogen is of controversial importance. For synthetic substances, however, no argument exists—the likelihood of closely related substances (such as "error peptides") being present in impure preparations, subsequently leading to the most objectionable variety of nonspecific antibody, means that maximum possible purity is essential. For particulate antigens, especially bacteria, there is also no reason for lack of purity, but the needs in respect to soluble substances extracted from natural sources are somewhat different. There is no doubt that relatively crude preparations are highly immunogenic, often more so than purer materials, so that many workers have thought of the impurities as having some adjuvant-like activity. The probability, however, is that greater purification has led to concomitant subtle chemical changes (such as deamidation) so that the immunogen stimulates antibodies that fail, to a greater or lesser extent, to "see" the native antigen. Despite this, a high degree of purification of immunogen must sometimes be sought in order to eliminate certain types of cross-reactivity in antisera. At the other extreme, gross impurity should be avoided, even when the cross-reactions are unimportant, because antigenic competition may then prevent formation of any specific antibody. In practical terms, about 10% purity is the minimum required to make a significant specific antibody response reasonably likely.

[2] J. J. Pratt, *Clin. Chem.* **24**, 1869 (1978).

The Adjuvant

A wide variety of substances are known to have the property of potentiating the humoral antibody response to injected immunogen. Among them are inorganic adsorbents, such as aluminum hydroxide gel; mineral oils, such as liquid paraffin; and bacterial cell wall components. The diversity of materials having adjuvant properties, which has been the subject of a recent review by Whitehouse,[3] makes it difficult to identify a simple mechanism of action. Three major effects are involved, albeit to different degrees for each adjuvant type. First, the release of immunogen from the site of injection is slowed, either by adsorption to solid particles or by incorporation into an oily emulsion. This leads to a "sustained release" from a depot at the injection site, where labile immunogens are also protected from breakdown by tissue enzymes. A secondary benefit is that any direct toxic effects of the immunogen on the recipient will be minimized. Second, adjuvants have a stimulatory effect on reticuloendothelial cells, attracting a local infiltration of the injection area by mononuclear cells and stimulating phagocytosis by macrophages presumably by presenting soluble immunogen in a particulate or partially aggregated form. Adjuvant-treated macrophages with antigen inoculated into histocompatible recipients give rise to a higher antibody response than transfer

[3] M. W. Whitehouse, *in* "Immunochemistry: An Advanced Textbook" (L. E. Glynn and M. W. Steward, eds.), p. 571. Wiley, New York, 1977.

of macrophages containing antigen alone.[4] Third, it has been shown that adjuvants induce an increased circulation of lymphocytes through lymphoid tissues in the drainage area,[5] macrophages being important for the initiation of these lymphocyte traffic changes.[6] The increased flow of cells in regional lymph nodes is likely to allow greater contact between antigen and antigen-reactive cells, thus facilitating increased antibody production. The local granulomatous lesions formed at the sites of injection may also serve as foci of antibody production.

The most important advance in adjuvant technology arose from the observation of Dienes and Schoenheit[7] that antigen injected into tuberculous granulomata stimulated higher antibody titers than did antigen injected at nontuberculous sites. These findings led Freund and co-workers to develop a series of adjuvants containing mycobacteria, mineral oil, and emulsifier,[8] which to this day remain the most potent tools available to the aspiring immunologist. The mixture most widely used in the preparation of reagent antibodies contains 9 parts of mineral oil to 1 part of detergent. The detergent (usually Arlacel A) contains a high level of both hydrophilic and lipophilic groups, thus facilitating dispersion of the oily and aqueous (immunogen) phases and allowing the formation of a stable emulsion. The simple oil–detergent mixtures are termed "incomplete" Freund's adjuvant; incorporation of heat-killed *Mycobacterium tuberculosis* or *M. butyricum* (0.5 mg/ml) into the oily mixture yields "complete" Freund's adjuvant. The latter is the more effective, probably as a result of greater stimulation of the local cellular response, and must now be regarded as an essential aid to the production of reagent antibodies against soluble immunogens.

Preparation of Freund's Emulsions. For maximum efficiency, it is necessary to obtain a stable, water-in-oil emulsion. Several ways of preparing such emulsions have been described, but there is no doubt that the simplest and most efficient, at least for the relatively small volumes that most people require, is the double-hub connector method described here. It may occasionally be difficult to persuade the phases to combine as water in oil rather than oil in water or mixed emulsions. Cooling the separate phases before mixing may help, but an infallible way of overcoming the problem is to use 2–4 volumes of oily adjuvant to 1 volume of aqueous

[4] E. R. Unanue, B. A. Askonas, and A. C. Allison, *J. Immunol.* **103,** 71 (1969).

[5] P. Frost and E. M. Lance, *in* "Immunopotentiation," CIBA Found. Symp. 18 (New series), p. 29. Excerpta Medica, Amsterdam, 1973.

[6] P. Frost and E. M. Lance, *Immunology* **26,** 175 (1974).

[7] L. Dienes and E. W. Schoenheit, *J. Immunol.* **19,** 41 (1930).

[8] J. Freund and K. McDermott, *Proc. Soc. Exp. Biol. Med.* **49,** 548 (1942).

immunogen. Experience has shown these oil-rich emulsions to be at least as effective (probably more effective) than the 1:1 ratio usually recommended. A subsidiary advantage is that they flow more easily, so both mixing and injection are less of a chore. If it is essential to use a 1:1 ratio (because of volume restrictions when the immunogen solution is very dilute, for instance), the formation of water in oil emulsions can be reliably achieved by adding the aqueous phase in three increments, mixing after each.

The necessary apparatus consists of the double-hub connector and two syringes, each large enough to contain the total emulsion volume without overfilling. The largest practicable volume that can be handled by someone with averagely large, reasonably powerful hands is 14–16 ml, using two 20-ml syringes. The best type of syringe for the purpose is an all-glass, center-hub pattern; metal-and-glass types tend to leak at the piston, and the common plastic syringes become very stiff while making the emulsion. Plastic syringes are reasonably satisfactory in the smaller sizes, however, since less force is needed for small volumes.

If Freund's complete adjuvant is required, shake it very thoroughly to resuspend the bacterial cells immediately before use. Pour out sufficient of the adjuvant into a small beaker (to avoid contaminating the remainder) and draw the required volume up into one of the syringes. Attach the double-hub connector, and carefully expel all air until the oil rises up into the farther end of the connector. Draw the aqueous immunogen into the other syringe, remove the needle, and again expel all air until the syringe hub is full of liquid, then connect it to the open end of the connector: any air left in the apparatus will be trapped in the emulsion and, because of its compressibility, will make injections more difficult. At this stage make sure that both syringes are firmly inserted into the connector, but be careful from now on not to place any bending stress on the rather unwieldly apparatus, especially if the syringes have glass hubs. A little oil will almost certainly be squeezed out of the connector (the whole process is somewhat messy) and it may be as well to wipe the apparatus and fingers with a tissue before proceeding.

To form the emulsion, begin by squirting the aqueous phase into the oil as vigorously as possible, then continue squirting the total contents to-and-fro from one syringe to the other a minimum of 10 times each way (20 times is better, if your thumbs can stand it). To avoid bending stress at the connections, practise deliberate relaxation of the "receiving" hand so that the filling syringe just rests on the palm as the other hand is grasping and pressing on the plunger. Especially as the hands tire it is tempting to let the receiving hand try to help the other, but this inevitably places strain on the syringe hubs. A fracture of a hub (or sudden falling apart of a

arelessly made connection) causes an explosive shower of emulsion to ontaminate everything with a radius of several feet (including the opera-or's face) and is sufficiently unpleasant to encourage more care thereaf-er.

If the aqueous phase is to be added in several aliquots in order to pro-note the formation of water-in-oil emulsions at the 1:1 ratio, it will be iecessary to break one of the connections each time more of the water hase is needed. About five each-way strokes of the syringe will be suffi-ient for the intermediate mixing, after which the next aliquot of immuno-en is taken up into the empty syringe, the connection is made again and, is before, mixing begins by squirting the water into the oily emulsion. Re-eated disconnection and reconnection make it all the more difficult to ex-lude air and prevent messy oil from leaking out: it is better to use a larger il to water ratio and avoid the problem altogether whenever possible.

In the authors' experience, the above method will lead infallibly to the roper type of emulsion, and testing is therefore unnecessary. For those vho wish to confirm success, however, the simplest way is to take a eaker half full of water and drop two small, separate drops of emulsion nto the water surface. The first drop always spreads somewhat, but the econd will remain a discrete, white globule with no spreading at all if the mulsion is, indeed, water in oil. If the second drop disintegrates into bits nd pieces that spread around over the surface of the water, the emulsion vas oil in water, at least in part, and should be prepared afresh. Read the bove instructions again first, though.

After use, plastic syringes should be thrown away, but other apparatus nust be washed up. The connector can first be pushed into a piece of rub-er tubing connected to a hot tap and flushed through for a few minutes. yringes should be cleaned with washing-up detergent, then soaked, to-ether with the connector, in a decontaminating detergent (such as Decon 0) for a day or two before rinsing and drying. Residues from emulsions re probably difficult to remove completely, and the syringes should ever again be used for any purpose other than preparing such materials.

The Choice of Animal

There are few instances in which categorical evidence has shown one pecies of common laboratory animal to give consistently better re-ponses than another to any particular immunogen. Some fairly well now exceptions are the superiority of guinea pigs for production of anti-nsulin sera (presumably because the endogenous hormone in this species s most unlike the other mammalian insulins) and of horses for preparation f antisera for immunoelectrophoresis. The latter preference is due to the

solubility of horse antibody immune precipitates in excess antibody (all immune precipitates are soluble in antigen excess) yielding unusually nar-row, clear-cut precipitin arcs. Apart from these examples, however, the literature abounds with indications of the personal preferences of the au-thors for which the evidence is apocryphal and often contradictory.

In most instances the choice of species may reasonably be made on the basis of what is available and the volume of antiserum required—the larger the animal, the bigger the yield. It will be understood that it is usually sensible to immunize a species that is "foreign" to the antigen in question. If homologous immunogens are used, it should be for a valid reason (production of tissue typing sera, for instance, relies on antibodies produced in the same species as the donor). Homologous immunization, when it produces a result at all, will yield antibodies that recognize fine, interindividual differences in the antigen; by contrast, immunization of a foreign species readily yields much more abundant antibody but any reac-tivity with the structurally minor, idiotypic variants of the antigen is al-most always lost in the reactivity against the gross, interspecies differ-ence.

A well provided laboratory may have access to guinea pigs, rabbits, sheep or goats, donkeys, and horses. There is little doubt that rabbits should be the first choice for most purposes unless very large amounts of serum are needed. Rabbits are cheap, easy to care for, robust in the face of quite intensive immunization, and easy to bleed. The other species may best be held in reserve in case of a failure with rabbits. Another reserve species that may be available is the chicken—again quite easy to handle, but producing antibodies that behave differently from those of mamma-lian species[9] and hence best avoided if possible.

Whichever species is chosen, it pays to immunize several individuals (which is a good reason for avoiding the larger, more expensive species to begin with). Individual variation in response is often very striking, espe-cially to the more "difficult" immunogens, and groups of at least four or five animals should be started if any difficulty whatsoever is to be ex-pected in preparing satisfactory antisera. Nonproductive animals can be disposed of once it is clear that they will not improve (this may not be for several months with some immunogens) whereas the better responders can be kept under immunization for a year or more and bled repeatedly. Obviously, such a course cannot be followed where the early antibody is desired, for instance in the production of hemolytic serum with minimal hemagglutinating activity for use in complement fixation tests.

[9] A. A. Benedict, *in* "Methods in Immunology and Immunochemistry" (C. A. Williams and M. W. Chase, eds.), Vol. 1, p. 229. Academic Press, New York, 1967.

Immune responsiveness to certain antigens has been shown to be genetically determined.[10] The importance of this in the context of antigens of general interest is not known, but it would seem to be desirable to use random-bred animals whenever possible, to give the best chance of a good response in one or more, unless previous experience has already shown that a particular inbred strain responds well to the immunogen in question. Whatever animals be chosen, they should be kept clean, healthy, and well fed if they are to perform well as antibody factories. The subject of animal husbandry is dealt with in a number of works (see, e.g., Short and Woodnott[11] and Chase[12]) but is, perhaps, of no direct interest to the readers of this chapter.

The Route of Injection

For soluble immunogens, it is generally believed that the efficiency of stimulation of the immune response is related to the site of inoculation. A probable series, in order of increasing effect, is intravenous < intramuscular < subcutaneous < intraperitoneal < intradermal < intraarticular < intranodal. The principal reasons for the differences in efficiency are the speed with which antigen is lost from the site of injection and the likelihood of it passing through the lymph nodes or other centers of immunological activity on the way. These considerations, however, are radically affected by the use of adjuvants, especially oily adjuvants, which may stimulate a brisk local cellular reaction and release antigen over a period of several weeks or even months.

Using oily adjuvants, then, the injection site can be chosen principally with a view to minimizing discomfort to the animal. Generally this means intramuscular injections in rabbits and larger animals or subcutaneous injections in guinea pigs; note that water in oil emulsions must never be given intravenously because of the virtual certainty of fatal fat embolism. Subcutaneous or intradermal injection of Freund's emulsions almost invariably leads to ulceration, but provided the sites are well chosen (see below) rabbits and guinea pigs show no sign of distress or loss of condition. Some authors (see Herbert[13]) have suggested that Freund's emulsions should not be injected subcutaneously since ulceration may lead to

[10] I. Green, W. E. Paul, and B. Benacerraf, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1095 (1969).

[11] D. J. Short and D. P. Woodnott, eds., "The I.A.T. Manual of Laboratory Animal Practice and Techniques," 2nd ed. Crosby Lockwood, London, 1969.

[12] M. W. Chase, *in* "Methods in Immunology and Immunochemistry" (C. A. Williams and M. W. Chase, eds.), Vol. 1, p. 254. Academic Press, New York, 1967.

[13] W. J. Herbert, *In* "Handbook of Experimental Immunology" (D. M. Weir, ed.), 2nd ed., App. 2. Blackwell, Oxford, 1973.

loss of the depot: in the experience of the present authors this has never given rise to difficulty. Occasionally deep abscesses form after intramuscular injection and lead to loss of condition. The abscesses are frequently "sterile" and, in our experience, have usually been related to overzealous attempts to improve on sterile injection techniques (cleaning the skin over the injection area, for instance) rather than to the use of unsterile immunogens.

Difficulties in preparing antisera against some of the antigens of interest in radioimmunoassay have led people to try a wide variety of methods of immunization. Most of these variations have been irrational (which does not mean to say they have not worked on occasion), but two deserve special mention. By injection of immunogen (angiotensin I, adsorbed on carbon black and emulsified in Freund's adjuvant) directly into rabbit lymph nodes and spleen, Boyd and Peart[14] obtained improved results that they believed to be due to more direct stimulation of the immune system. A subsequent comparative trial gave rather equivocal results,[15] however, and the method was too difficult to be widely used. Injection into the Peyer's patches (lymphoid patches in the intestinal wall, quite easily visible in the rabbit) is technically much simpler but has proved no more successful in the authors' hands.

Much simpler than the intranodal method, and now quite widely used, is the method of multiple intradermal inoculation introduced by Vaitukaitis *et al.*[16] The immunogen is introduced at 40 or more sites spread widely over the body surface. Antibody response to this primary immunization is much greater than to a first injection given in the usual way, and no more than one booster injection is usually required. Comparison with the usual intramuscular injection schedule[1] showed no great difference in efficiency, although the multiple intradermal technique (with only one booster) required rather less immunogen and yielded effective antisera in a shorter period of time.

The Dosage of Immunogen and Timing of Injections

Although an animal may be made "tolerant" to soluble antigens given in too low or too high a dose under certain circumstances, the use of a potent adjuvant makes such an outcome extremely unlikely. Nevertheless, the observation that too high a dose can lead to antiserum of rela-

[14] G. W. Boyd and W. S. Peart, *Lancet* **2**, 129 (1968).

[15] B. A. L. Hurn and J. Landon, *in* "Radioimmunoassay Methods" (K. E. Kirkham and W. M. Hunter, eds.), p. 121. Churchill Livingstone, Edinburgh, 1971.

[16] J. Vaitukaitis, J. B. Robbins, E. Nieschlag, and G. T. Ross, *J. Clin. Endocrinol.* **33**, 988 (1971).

tively low avidity,[17,18] presumably owing to stimulation of lymphocytes bearing low-affinity receptors, may certainly be relevant even when using Freund's adjuvant. Since most sensitive immunoassay techniques of current interest rely on antibody of the highest possible avidity, it is evidently desirable (and economical of immunogen) to use the lowest dose that will be fully effective. This dose is very much smaller than most of the published literature recognizes, and a suitable priming (first) inoculation for rabbits or guinea pigs will generally be of the order of 100 μg. A range of 50–1000 μg should cover all needs, depending on the purity and immunogenicity of the material in question (but it is sensible to start at the lower end, since an animal showing lack of response after a sufficiently long trial can then be given a larger dose, whereas an animal producing poor antiserum after high dosage is beyond hope of salvage). The dosage required for larger animals does not increase in proportion to body weight: 0.25–5 mg is satisfactory for sheep and 0.5–10 mg for donkeys. For conjugated haptens, incidentally, these figures refer to total conjugate weight.

Booster injections are always needed to obtain antisera of the highest titer and avidity. Practical experience suggests that good results will be obtained using a booster dose about half the size of an effective priming dose, given by the same route (not necessarily at the same site) and using Freund's complete adjuvant on each occasion. It is recognized that these recommendations are somewhat at variance both with immunological theory (which would suggest a progressive increase in dose) and with the advice of other authors to avoid repeated use of Freund's complete adjuvant, especially subcutaneously, because of abscess formation and hypersensitivity reactions. There is some documented evidence in support of the suggested reduction in dose,[1] but the repeated use of complete Freund's adjuvant is a recommendation that stems only from satisfactory, albeit uncontrolled, experience.

The repeated booster doses that are usually required for the best antiserum should not be given too frequently. It has been shown[19] that no further rise in titer results from a second injection given before the response to the first is reaching its peak. At least 4 weeks should pass between injections of Freund's emulsions. After the first booster, or sometimes after the second, antibody response may be quite prolonged and many people believe that a rest of 3–6 months is desirable before the next injection if antiserum of the highest avidity is required; the evidence in favor of this approach is not strong,[1] but in general terms there is little doubt that patience is desirable when making reagent antibodies. It is not unusual to read descriptions of immunization schedules involving weekly injections of quite large amounts of immunogen in Freund's emulsion; published accounts, not surprisingly, tend to report a successful outcome, but the approach is not to be recommended.

[17] G. W. Siskind and B. Benacerraf, *Adv. Immunol.* **10**, 1 (1969).
[18] E. J. Greene and J. G. Tew, *Cell. Immunol.* **26**, 1 (1976).
[19] W. J. Herbert, *Immunology* **14**, 301 (1968).

Many published immunization procedures terminate with one or more intravenous injections of soluble immunogen given without adjuvant after a course of intramuscular Freund's emulsions. In the authors' experience, this produces a less satisfactory response (about half the final titer of avid antibody) than can be obtained with a final injection of intramuscular emulsion.

By contrast with the above, particulate immunogens are normally administered intravenously, frequently (perhaps every other day), in increasing doses and for short periods of time. These materials are usually highly immunogenic, partly because the normal mechanism for their removal brings them into close contact with the immune system and partly because many of them (notably bacterial cells) are antigenically very "foreign" to the immunized animal. Antibody production is rapid, and the early IgM response is excellent for agglutination tests. Initial doses of immunogen are extremely variable, owing to the variable toxicity of the substances concerned (especially bacteria containing endotoxins), and for many of the antigens hypersensitivity reactions to later doses may prove rapidly lethal. Subcutaneous injection, with relatively slow absorption, may ameliorate undesirable acute reactions.

Although short immunization courses for particulate antigens are the rule, usually in the belief that antisera will become less specific as immunization proceeds, this is not necessarily the case. Prolonged immunization may result in more stable IgG antibody of higher titer and, because of repeated bleeding over a period of time, in much greater yield.

Practical Immunization Schedules

Animals often remain under immunization for many months, even years. You may not be personally responsible for their care during this time, but in your own interests you must ensure that either the individual animals or their cages are properly labeled in a manner compatible with your own records at the time of the first injection so that the individual animals can be identified with certainty thereafter. If the cages alone are labeled, you would also be advised to ensure that the method of animal handling, especially during cage cleaning, is such as to prevent animals being moved accidentally from one cage to another.

Rabbits

Four or more healthy, young adult rabbits should be treated with each immunogen.

Soluble Immunogens

Either the intramuscular or the multiple intradermal route may be recommended. As examples of representative immunogens for which high-avidity antisera are required, consider a crude preparation of human chorionic gonadotropin (hCG) and the beta subunit of hCG (β-hCG). The former, at a characteristic potency of 1500–3000 IU/mg, is about 20% pure whereas the latter is of necessity highly purified and in short supply. Appropriate doses for primary immunization are 1 mg and 100 μg, respectively. Booster doses should be half these amounts.

Dissolve the immunogen in isotonic saline (other immunogens may require slight acidity, alkalinity, or other special condition) to a volume of 0.5 ml per rabbit for the primary injection or 0.25 ml for boosters (i.e., the same concentration for both injections). Emulsify the solution with three volumes of Freund's complete adjuvant, using a double-hub connector and two syringes as described above. The total volume of emulsion will then be 2 ml per rabbit for the primary inoculation or 1 ml for a booster. Use the emulsion within an hour of preparation.

Intramuscular Schedule. Do not shave the animals or attempt to prepare the skin in any way prior to injection. A fairly stout needle of medium length (21 gauge × 1 inch) is convenient and need not be changed between animals unless it becomes blunted for any reason. Injections are given into thigh and/or upper foreleg muscle, where thickest, and the hair can be parted by gently blowing down on to the selected site immediately before injection.

For the primary injection, give 0.5 ml of emulsion intramuscularly into each of the four limbs of each animal. Now go away and think about other things for *at least* 4 weeks, or 6 weeks if possible.

For booster injections, give 0.5 ml of emulsion intramuscularly either into each hind limb or into each fore limb, alternately. Bleeds (20–40 ml) may be taken for testing on two occasions between 7 and 10 days after each booster and similarly every 3–4 weeks thereafter if the antiserum is satisfactory. Further boosters may be given at *minimum* intervals of 4 weeks (but preferably not within 2 weeks of a bleed) although it may pay to rest the animal for 3–4 months after the second or third booster.

Animals that fail to show a reasonable response after two or three boosters should be disposed of. This decision must be related to the level of response expected for the particular immunogen used—some animals

may take several months to respond to "difficult" immunogens, and early responders are not necessarily the best in the end.

Multiple Intradermal Method. Shave the hair on the back and on the proximal parts of all four limbs of each rabbit. As a guide to spacing the injections, draw six transverse lines across the shaved area of the back, using a felt-tip marker. The injections should be made with a tuberculin syringe and a fine needle (the syringe holds only enough for one animal but may be loaded repeatedly from the syringe in which the emulsion has been prepared, via the double-hub connector).

Make 24 intradermal injections each of 0.05 ml, spaced evenly over the back. Distribute the remainder of the emulsion (about 0.8 ml, or sixteen 0.05 ml injections) over the inner and outer aspects of each upper limb, in the shaved areas. Satisfactory intradermal injections are easily recognized by a characteristic, localized bleb; this is easy to achieve on the back of the animal, where the dermis is quite thick and tough, but very difficult on the limbs, where the skin is much more delicate. Try, but do not be unduly discouraged if you fail.

Within a few days of the injections the rabbit will present a horrifying sight, covered as it will be with forty, half-inch ulcers. In the authors' experience, the animals are happily unaware of the aesthetics of the situation and continue to thrive without any specific treatment. Some users of the technique have found otherwise, for no known reason. In the interest of animal welfare, if you find your rabbits are greatly upset by this procedure then please revert to the intramuscular procedure, which can be just as effective.

After the multiple injections the animals should be left for *at least* 10 weeks before boosting. Antibody levels rise to relatively high titers during this time, however, and it is certainly worth taking a large bleed for testing after 8–10 weeks. All booster injections are given by the intramuscular route, and the method of treatment from the tenth week onward is thus exactly the same as for the previous schedule.

Particulate Immunogens

These antigens are commonly administered by frequent, intravenous injection without adjuvant. Results are obtained quickly, the antisera often containing a high proportion of IgM immunoglobulin. There is a risk both of direct toxicity early in immunization and of severe hypersensitivity reactions as a result of later injections. With some at least of the antigens in question, equally satisfactory results can be obtained by intramuscular injection of Freund's emulsions—immunization is slower but less risky.

Production of antisera to *Escherichia coli*, for use as specific typing reagents, furnishes an example of a typical intravenous schedule. Good bacteriological technique and the selection of an appropriate colonial form of the organism is essential to the specificity and reactivity of the antiserum (this is obviously analogous to the purification of a soluble immunogen). Living organisms are required for expression of the important K antigens in this species, but live coli will kill a high proportion of unprotected animals and the early injections are therefore made with heat-killed suspensions. Antisera to most other microbial species can be prepared against killed suspensions throughout. The following schedule should be followed (all suspensions being prepared to an opacity of Brown's tube 4, and all injections given intravenously).

Day 1: 0.25 ml of killed suspension
Day 3: 1.0 ml of killed suspension
Day 5: 3.0 ml of killed suspension
Day 9: 0.5 ml of freshly prepared living suspension
Day 12: 1.0 ml of freshly prepared living suspension
Day 16: 3.0 ml of freshly prepared living suspension
Day 22: test bleed for titer
Either
Day 23 Bleed out if titer is satisfactory
Or
Continue weekly injections as for day 16 with test bleeds 5–7 days later, until satisfactory titers are obtained.

Guinea Pigs

Each animal will yield only 3–5 ml of serum by cardiac puncture or 15–25 ml when bled out. For this reason guinea pigs are best reserved for use when only small quantities of antiserum are required (particularly in radioimmunoassay and similar immunoassays) or when other animals are known not to respond well to the immunogen in question (insulin is such a substance, and, in our experience, parathyroid hormone is another). Groups of up to 10 guinea pigs may conveniently be kept in a single large cage, individuals being identified by natural markings or applied pigments (the latter need to be renewed rather frequently).

Soluble immunogens should be administered as Freund's emulsions, injected subcutaneously into the abdominal wall just on either side of the midline. The injection sites will usually ulcerate after a week or so, but the animals are apparently free from discomfort, thrive, and make good antibodies.

Prepare the inoculum by emulsifying 1 volume of aqueous immunogen in 2–3 volumes of Freund's complete adjuvant in the usual way, to give a total volume of 0.5 ml per animal. Injections should be given at intervals of not less than 4 weeks although longer rests later in the course of immunization may be desirable. Because of the low yield of serum and the risk of killing the animals when bleeding by cardiac puncture, it is less practicable to bleed guinea pigs repeatedly than it is to bleed rabbits. Since guinea pigs are cheaper to buy and look after, it is probably best to immunize a relatively large number for a comparatively long period of time, then bleed them out and select the best antisera from the result. Our experience has suggested that at least four injections are desirable if this strategy is employed, and six injections may often be better. The decision depends on the purpose for which the antiserum is required and, in particular, whether the highest possible avidity is needed.

Sheep

The immunization of sheep offers the possibility of obtaining relatively large amounts of antiserum, not only because each individual bleed is larger (150–300 ml of serum, depending on the size of the animal), but also because the animals may be maintained and bled repeatedly for longer than rabbits. This can be a major advantage when antisera are to be prepared for relatively undemanding, insensitive test systems such as immunoprecipitation, when larger volumes of reagent are required but variations in quality over the course of time are unlikely to cause difficulty. The higher cost of buying and keeping a sheep makes it less attractive when the use of a "difficult" immunogen makes it necessary to immunize a large number of animals. Circumstances alter cases, of course, and an Australian laboratory might have a different view of the relative economy of sheep and rabbits.

Immunization of a sheep should proceed according to a schedule similar to that described for a rabbit. Intramuscular injections (as usual, always prepared with Freund's complete adjuvant) should be given with a 1½–2-inch needle deeply into the haunch or shoulder (preferably into all four "corners" for the first injection). As has been mentioned before, dosage is not proportional to size and for a relatively good immunogen such as human IgG an initial injection of 0.2–1 mg, followed by booster doses of half that size, should be sufficient. After the first two or three monthly injections, subsequent boosts should be given at longer intervals depending on the quality of antiserum. Bleeds may be collected on a regular schedule throughout the period of immunization, the best yields being obtained if three bleeds (of 300–600 ml, depending on the size and experience of the animal) are taken over a period of 8–10 days followed by

ut 3 weeks rest before the next triple bleed. A healthy animal may re-in productive for some considerable time, at least a year or two, but ibody levels will eventually decay and fail to respond to a further oster injection, at which time the animal should be disposed of.

Collection and Storage of Immune Serum

Animals immunized with Freund's emulsions should be bled 7–10 ys after booster injections. If the blood is taken from a vein rather than cardiac puncture, two or three bleeds can be taken on successive days, the animal should then be rested for 3–4 weeks before further bleed-: or before boosting again if the original antiserum was not of satisfac-y quality. After intravenous injection antibody levels rise and then fall re rapidly and bleeds should be collected 5–7 days after the last dose. s often helpful to fast the animals overnight to minimize lipemia, but do deprive them of drinking water.

Blood should be collected in clean, dry, glass bottles and allowed to t at room temperature or at 37° until the clot has retracted; it may help "ring" the clot with a glass rod to promote separation. The sample uld then be centrifuged and the serum be separated without undue lay in order to avoid unnecessary hemolysis, which looks unaesthetic hough it has no obvious deleterious effect on the antibody. When han-ng large quantities of blood it may be easier to separate serum from the t by letting it drain through a stainless steel mesh cone supported in a er funnel—this can even be left to drain overnight in the cold room if maximum possible yield is required, but in any case a final centrifuga-n will be required to remove residual red cells.

After separation from the clot, antiserum may be stored without signif-nt deterioration for long periods of time under a variety of conditions.[20] counsel of perfection for reference or otherwise most precious reagents uld be to filter sterilize, fill out in appropriate, accurately measured, all amounts (diluting in a suitable carrier medium if necessary), and n freeze-dry prior to storage at 4° or below. Experience has shown, wever, that IgG antibodies are remarkably robust and that liquid anti-um (even without sterilization) can be kept for many months at 4° with % sodium azide added as an antibacterial agent. Storage at about −20° he ordinary laboratory freezer cabinet is, in theory, likely to cause pro-n denaturation due to the proximity of this temperature to the eutectic sodium chloride (the complex mixture comprising serum will not be

. E. Kirkham and W. M. Hunter, eds., *in* "Radioimmunoassay Methods," pp. 189–193. hurchill Livingstone, Edinburgh, 1971.

completely frozen at −20°) but, again in practice, the freezer has proved most convenient and harmless to antibody protein provided that repeated freezing and thawing is avoided. Storage at lower temperature, preferably not in unreliable mechanical refrigerators, is very satisfactory when available. Gas-phase liquid nitrogen is the ideal low-temperature storage medium, being more reliable and convenient than mechanical or CO_2 cabinets, not involving the special restrictions on storage vessels imposed by immersion storage in liquid nitrogen yet virtually guaranteeing lifetime stability of precious antisera (the investigator's lifetime, that is to say).

Storage of IgM antibodies is far more of a problem and gives very variable results. Most antisera containing IgM can be handled exactly as described above, with only gradual deterioration that would be inapparent in the relatively undemanding test systems in which this class of antibody is generally used. Some, on the other hand, prove much less stable. On occasions, this instability is associated with bacterial growth (which seldom causes much loss of IgG antibody activity although it is embarrassing and should be avoided if possible). For this reason it is strongly recommended that IgM antisera should have 0.1% sodium azide added, be sterilized by filtration at the earliest possible opportunity (before bacterial growth and release of enzymes can occur) and be handled in a cleanly fashion thereafter.

Even when collected after overnight fasting of the animal, defatted (see below), sterilized and with a bacteriostat added, serum stored at 4° will gradually become turbid and show a deposit, principally of denatured lipoprotein. This does not lead to any loss of antibody activity although it is easily mistaken for bacterial contamination and causes anxiety for that reason. The only practical disadvantage is seen when the antiserum is used in capillary precipitin reactions, when the turbidity can obscure the result unless the antiserum is first clarified by filtration.

Further Treatment of Antisera

Defatting Antiserum[21]

Antisera to be used in capillary precipitin tests must be crystal clear so that the faint ring of precipitation can be easily seen. Untreated sera become turbid on storage, due to precipitation of denatured lipoprotein; such precipitates can be removed by membrane filtration prior to use, but it is usually better to reduce the severity of the problem by extracting the bulk of the lipoprotein at the time the serum is first prepared.

[21] A. S. McFarlane, *Nature (London)* **149**, 439 (1942).

Materials

Diethyl ether, solvent grade
Solid CO_2–methylated spirit freezing bath

Procedure

1. Place the serum in a beaker and add 3 ml of ether for every 10 ml of serum.
2. Place the beaker in the freezing bath.
3. Stir the serum–ether mixture quite briskly with a glass rod until it has frozen solid. The two liquids are completely miscible in these proportions at the freezing point.
4. Allow the frozen mixture to stand in the freezing bath for another 10 min, then remove the beaker and stand it in tepid water until the frozen plug loosens.
5. As soon as possible, tip the still frozen plug into a glass filter funnel (without filter) leading into a cylindrical separating funnel. Make sure the stopcock on the latter is free running and well lubricated with a silicone grease.
6. Allow the frozen material to thaw and run into the separating funnel at room temperature, then remove the filter funnel and close the separating funnel with a rubber stopper covered in metal foil.
7. Allow the separating funnel to stand undisturbed, at 4° if possible, overnight.
8. The next day the serum–ether emulsion will have separated into a lower layer of clear serum shading gradually into an opalescent zone of residual emulsion that has a sharp interface with the uppermost, opaque, fatty layer. Collect the serum by running off the bottom and intermediate layers.
9. Remove the bulk of the residual ether by boiling off under reduced pressure, ideally with the aid of a rotary evaporator.
10. Add preservative and sterilize the serum by filtration prior to storage.

NOTE: Due care should be taken to avoid the risk of fire or explosion when handling ether.

Absorption of Nonspecific Antisera

The production of potent antiserum almost always results in a reagent with some degree of reactivity against nonspecific antigens, either because of impurities in the immunogen used or because there are "shared determinants" present in both specific and nonspecific antigens. Whatever the cause of the unwanted reactivity, it is usually necessary to remove it by absorbing the antiserum with an appropriate antigen. Although absorption may be carried out with solutions of the antigen (the immune precipitate being removed afterward by centrifugation or filtration), excess antigen or soluble complexes of antibody and antigen will inevitably remain in the absorbed antiserum. A more satisfactory method, therefore, is the use of a solid phase immunoadsorbent prepared from the appropriate antigen, which can be added in excess and easily recovered for later re-use if required. Such adsorbents may be made from antigen alone by the use of cross-linking reagents (such as glutaraldehyde for protein antigens) or can be more complex reagents prepared by chemical coupling of the antigen to a solid support such as Sepharose. The latter technique is covered in a subsequent section on the use of IgG–Sepharose immunoadsorbent, but the present example describes the preparation of a glutaraldehyde polymer of $F(ab')_2$ suitable for removal of light-chain cross-reactivity from class-specific anti-immunoglobulin sera. It should be noted that the pH optimum for efficient polymerization by this method varies considerably depending on the protein to be treated; if a polymer of whole serum is required, for instance, a pH of 4.4 will be optimal.

Preparation of $F(ab')_2$ Immunoadsorbent by Glutaraldehyde Polymerization[22]

Materials

$F(ab')_2$ prepared by pepsin digestion of IgG[23]
Phosphate buffer, 0.1 *M*, pH 7.0
Glutaraldehyde, 2% in saline
Glycine-HCl buffer, 0.1 *M*, pH 2.5
Tris-HCl, 0.1 *M*, pH 8.0
Phosphate-buffered saline, 10m*M*, pH 7.5 (PBS)

Procedure

1. Dialyze 100 mg of $F(ab')_2$ preparation (20–50 mg/ml) against phosphate buffer at 4° overnight.
2. Place the $F(ab')_2$ solution on magnetic stirrer and add 0.4 ml of glutaraldehyde solution dropwise from a Pasteur pipette.
3. Allow the gel that forms to remain at room temperature for 3 hr and then place at 4° overnight.
4. Homogenize the gel in phosphate buffer then centrifuge hard in a bench centrifuge and discard the supernatant.
5. Repeat step 4 using glycine-HCl buffer.

[22] S. Avrameas and T. Ternynck, *Immunochemistry* 6, 53 (1969).
[23] L. H. Madsen and L. S. Rodkey, *J. Immunol. Methods* 9, 355 (1976).

6. Repeat step 4 using Tris-HCl buffer.
7. Repeat step 4 using PBS.
8. Wash polymer in PBS until the washings have negligible absorption at 280 nm.

Tube Absorption Procedure

1. Mix 2 volumes of serum with 1 volume of packed polymer and stir on a magnetic stirrer at 37° for 1 hr.
2. Centrifuge at 4500 rpm for 5 min.
3. Transfer supernatant to another tube and recentrifuge.
4. Remove the supernatant and test it for specificity.

NOTE: The polymer can be "regenerated" for further use by washing extensively with PBS followed by incubation with 3 *M* sodium thiocyanate, pH 6.6, for 30 min at room temperature to elute adsorbed protein. Wash the polymer finally with PBS and store at 4° in PBS containing 0.1% sodium azide.

Preparation of Immunoglobulin Fractions from Whole Serum

Precipitation with Rivanol and Ammonium Sulfate

Materials

Rivanol (2-ethoxy 6,9-diaminoacridine lactate)
Activated charcoal
Saturated ammonium sulfate solution
Isotonic saline

Procedure

1. Adjust antiserum to pH 8.5 by careful addition of 0.1 *N* NaOH.
2. For each 10 ml of antiserum add 35 ml of 0.4% Rivanol solution dropwise from a separating funnel. Stir the serum gently on a magnetic stirrer throughout.
3. Decant the supernatant (containing the immunoglobulins) into universal bottles and centrifuge in a bench centrifuge to remove remaining sediment.
4. Decant the supernatant into a conical flask and add activated charcoal (1–1.5 g per 100 ml) to decolorize the solution. Agitate gently for approximately 10 min.
5. Remove charcoal from the protein solution by filtering through a double layer of moistened filter paper (Whatman No. 42) in a Büchner funnel. Transfer filtrate to a beaker.
6. Add an equal volume of saturated ammonium sulfate solution dropwise from a separating funnel, stirring gently on a magnetic stirrer throughout.

7. When all the ammonium sulfate solution has been added, place the beaker at 4° for at least 6 hr to allow the immunoglobulin precipitate to flocculate.
8. Centrifuge at about 4000 *g* for 20 min, preferably in a refrigerated centrifuge, and discard the supernatant.
9. Dissolve the precipitate in a volume of saline approximately equivalent to half the volume of original antiserum.
10. Place the immunoglobulin solution in Visking tubing and dialyze extensively against several changes of saline to remove sulfate ions. (Alternatively, remove sulfate by chromatography on a Sephadex G-25 column.)
11. Check for residual sulfate ions by adding a few drops of the immunoglobulin solution to a tube containing a small volume of barium chloride solution. Any cloudiness indicates the presence of sulfate ions and the need for further dialysis.
12. Measure the volume of immunoglobulin solution and calculate the protein concentration by measuring the absorbance of a 1:25 dilution at a wavelength of 280 nm using a cuvette of 1 cm path length.

$$\text{concentration} = (OD_{280} \times 25)/1.34 \text{ mg/ml}$$

(The factor 1.34 can be used for the immunoglobulins of most animal species).

Precipitation with Caprylic Acid[24]

Materials

Acetate buffer, 60 m*M*, pH 4.0
Caprylic acid
Isotonic saline

Procedure

1. Add 2 volumes of acetate buffer to the antiserum in a beaker. Check and adjust the pH of the mixture to 4.8.
2. For each 10 ml of starting antiserum add 0.74 ml of caprylic acid dropwise. Stir the mixture continuously on a magnetic stirrer at room temperature.
3. Continue stirring for 30 min.
4. Centrifuge at 4000 *g* to remove the precipitate (or filter on a Büchner funnel).
5. Retain the supernatant (containing the immunoglobulin) and dialyze extensively against saline at 4°.

[24] N. Steinbuch and R. Audran, *Arch. Biochem. Biophys.* **134**, 279 (1969).

6. Measure the volume of the immunoglobulin solution and calculate the protein content as described above.

NOTE. Since the final volume of immunoglobulin solution is approximately three times the volume of starting antiserum, concentration is usually necessary. This may be achieved by ammonium sulfate precipitation as described above, pressure ultrafiltration, or dialysis against hypertonic polyethylene glycol. If the latter procedure is used, the immunoglobulin preparation should subsequently be redialyzed against saline to remove any polyethylene glycol that has diffused into the dialysis bag, thereby contributing to the absorbance at 280 nm.

Ion Exchange Chromatography

Immunoglobulins, in particular IgG, may be separated from whole serum by ion exchange chromatography. The technique relies upon differences in the net charge of serum proteins: at low ionic strength and neutral pH, IgG carries a neutral or slight net positive charge and will not be adsorbed to diethylaminoethyl (DEAE) cellulose, unlike all other serum proteins. Although the principle of the method remains the same for the serum proteins of different species, the exact conditions of pH and ionic strength required for good separation of IgG will vary. A method for preparation of rabbit IgG by ion exchange chromatography using a batchwise procedure is outlined below.

Materials

Diethylaminoethyl (DEAE) microgranular preswollen cellulose (Whatman DE-52)
Phosphate buffer, 5 m*M* pH 6.5

Procedure. The batchwise procedure of Stanworth[25] is used.

1. Equilibrate approximately 5 g of DEAE-cellulose with several changes of phosphate buffer.
2. Dialyze 20 ml of serum against phosphate buffer at 4° overnight.
3. Place the cellulose slurry in suitable containers such as universal bottles or large test tubes and centrifuge to sediment the particles. Check the pH of the supernatant buffer against that of the starting buffer to ensure that equilibration is complete. Discard the supernatant.
4. Add dialyzed antiserum to the packed cellulose and mix by gentle rotation for 1 hr at room temperature.
5. Centrifuge gently to sediment the cellulose, then carefully transfer

[25] D. R. Stanworth, *Nature (London)* **188**, 156 (1960).

the supernatant (containing immunoglobulin) to a clean container. Discard the cellulose.

6. Recentrifuge to remove any remaining cellulose, and decant the supernatant immunoglobulin solution.
7. Calculate the protein content as described previously.

If this preparation contains serum proteins other than immunoglobulin the process may be repeated using a fresh aliquot of equilibrated DEAE-cellulose.

Preparation of Immunospecific (Affinity-Purified) Antibody

For some purposes it is necessary to use specific antibody rather than whole antiserum or a crude immunoglobulin fraction. Immunospecific antibody can be prepared by passing antiserum or a globulin fraction through an immunoadsorbent column containing antigen chemically coupled to an inert solid phase. Specific antibody combines with the immobilized antigen and can be eluted subsequently with "chaotropic" ions (such as thiocyanate) or low pH buffers. A method for preparation of human IgG immunoadsorbent and the elution of specific anti-IgG antibodies is given here.

Use of IgG-Sepharose Immunoadsorbent Prepared by Periodate Oxidation[26,27]

Materials

Sepharose CL4B
Sodium metaperiodate
Ethanediol
Isotonic saline
Carbonate–bicarbonate buffer, 0.1 *M*, pH 9.5
Phosphate-buffered saline 10 m*M*, pH 7.5 (PBS)
Sodium borohydride
Sephadex G-50, suspended in PBS
Sodium thiocyanate, 3 *M*, adjusted to pH 6.6

Procedure

ACTIVATION OF SEPHAROSE

1. Suck dry some of the Sepharose CL4B slurry. Weigh out 20 g of the gel and wash it with saline in a Büchner funnel containing two Whatman No. 54 filter papers.

[26] C. J. Sanderson and D. V. Wilson, *Immunology* **20**, 1061 (1971).
[27] T. J. G. Raybould and S. M. Chantler, *J. Immunol. Methods* **27**, 309 (1979).

2. Make up 40 ml of 10% sodium metaperiodate solution in distilled water.
3. Suck the Sepharose dry on the Büchner funnel and transfer the pad to the periodate solution. Mix or stir gently for 2–4 hr at room temperature.
4. Transfer the Sepharose slurry to a Büchner funnel containing two Whatman No. 54 papers. Wash quickly with saline to remove periodate.
5. Pour on 40 ml of 10% aqueous ethanediol, allowing the liquid to run through the gel very slowly to ensure thorough washing.
6. Wash the activated Sepharose finally with sodium carbonate–bicarbonate buffer and suck dry.

COUPLING OF ANTIGEN

1. Prepare 100 ml of IgG solution at a concentration of 1.0 mg/ml in sodium carbonate–bicarbonate buffer.
2. Add the activated Sepharose to the IgG solution and mix or stir gently for 18 hrs at room temperature (or 4° if preferred).
3. Transfer the slurry to a Büchner funnel containing two Whatman No. 54 papers. Suck dry and wash with PBS.
4. Prepare 20 ml of a 5 mg/ml aqueous sodium borohydride solution.
5. Transfer the Sepharose pad to the borohydride solution and mix or stir gently for 2 hrs at room temperature. (CARE: Borohydride reduction is accompanied by evolution of hydrogen and should be carried out in a loosely stoppered vessel in a well ventilated area).
6. Transfer the gel to a Büchner funnel containing two Whatman No. 54 papers and suck dry. Wash extensively with PBS, and finally resuspend in PBS to desired concentration. The gel is now ready for use.

PREPARATION OF IMMUNOADSORBENT COLUMN

1. Clamp a column (approximately 1.5 cm × 40 cm) to a stand, and with the outlet closed run a small volume of PBS into the column.
2. Pour the Sephadex G-50 suspension into the column and allow to settle until approximately 1 cm of column length is filled. Open the outlet to allow a flow of PBS, which facilitates column packing. Add more Sephadex slurry to give a packed volume of one-third of the column length, with a reasonable depth of PBS above the packed Sephadex. Close the outlet.
3. Pour the IgG–Sepharose slurry into the column carefully so as not to disturb the surface of the Sephadex and allow it to settle in a separate layer on top of the Sephadex.
4. Cut a circle of Whatman No. 54 filter paper the same size as the internal diameter of the column and allow to float onto the settled surface of the IgG–Sepharose. This prevents disturbance of the surface of the column during subsequent sample and buffer applications.
5. Open the column outlet to allow excess buffer to run through and wash the column contents by passage of PBS until the absorbance of the effluent at 280 nm is equivalent to that of washing buffer.

APPLICATION OF ANTISERUM OR GLOBULIN SAMPLE

1. Dialyze 1 ml of the serum or globulin solution against PBS overnight at 4°.
2. Open the column outlet to allow the head of buffer to pass into the gel. Close the outlet.
3. Apply the dialyzed sample to the top of the column, taking care to avoid disturbance of the Sepharose.
4. Open the outlet and allow the sample to run into the Sepharose column, closing the outlet when all the liquid has been absorbed.
5. Run PBS onto the top of the column and allow to flow through slowly by opening the outlet slightly. Ensure that a head of PBS is always present to avoid drying out.
6. Unadsorbed serum proteins will pass through the column and can be detected by a suitable monitor. When all the protein has emerged allow the remaining head of PBS to pass into the column and then close the outlet.

ELUTION OF BOUND ANTIBODY

1. Gently apply 5 ml of sodium thiocyanate solution to the column and allow to run into the gel by opening the outlet.
2. As soon as the thiocyanate solution has entered the gel, close the outlet, apply PBS, reopen the outlet and allow PBS to flow continuously through the column as previously. Eluted antibody contained in the thiocyanate solution will pass through the Sepharose and into the lower, Sephadex portion of the column. The molecular sieving properties of the Sephadex will serve to separate antibody rapidly from the thiocyanate and reduce the risk of denaturation.
3. Collect fractions containing the antibody, pool, and concentrate to approximately 5 mg/ml.
4. Measure the volume and protein content. Store frozen or freeze-dried in suitable size aliquots.

NOTE. This method of purification will select all antibodies reacting with the antigen on the immunoadsorbent, including any that may cross-react with other antigens by virtue of shared determinants. If such antibodies are likely to be present (as, for instance, will be the case in antisera raised against whole IgG), they should be removed by straightforward ab-

sorption as described in a previous section: Absorption of Nonspecific Antisera. Absorption may be carried out before or after preparation of the immunospecific antibody, but the former is to be preferred for logistic reasons.

Preparation of Fluorochrome and Enzyme-Labeled Antibodies

Selection of Antisera for Conjugation

Satisfactory conjugates can be prepared only from potent antisera of the required immunological specificity; it is important therefore that the purest available antigen be employed as immunogen and that multiple injections be given to ensure the production of antisera in which the ratio of antibody globulin to nonantibody globulin is high.

Ideally, antiserum should be selected on the basis of tests of potency and specificity carried out prior to labeling. This preliminary evaluation may conveniently be performed by titration in conventional gel diffusion and assessment of specificity in immunoelectrophoresis. If the lack of a suitable soluble antigen makes such tests impossible, indirect immunofluorescent or immunoenzyme tests should be done utilizing a range of dilutions of pre- and postimmunization sera as the intermediary layer followed by the appropriate labeled anti-species immunoglobulin. Test samples, which may be histological preparations or cell films should be appropriately prepared (some prior knowledge of the system is almost essential) and should represent both "positive" (antigen containing) and "negative" (non-antigen containing) materials. Antisera exhibiting the highest level of activity and specificity should be selected for labeling.

Labeling should be carried out on immunoglobulin preparations derived from the selected antisera, so as to maximize the proportion of specific antibody to total protein and hence reduce non-specific activity in the final reagent. Immunoglobulin can be prepared by any of the methods described above, but only in the most demanding systems will it be necessary to prepare immunospecific antibody rather than a crude immunoglobulin fraction.

Fluorescein Labeling of Antibody Globulins

Materials

Fluorescein isothiocyanate, isomer I (FITC)
Carbonate–bicarbonate buffer, 0.1 *M*, pH 9.0
Immunoglobulin preparation (10 mg/ml in saline)
Phosphate-buffered saline, pH 7.5 (PBS)
Sephadex G-50 medium

Procedure

1. Prepare a solution of FITC in carbonate–bicarbonate buffer to give a solution containing 1 mg of dye per milliliter.
2. Place a measured volume of the immunoglobulin solution in a small beaker and cool to 4°. Place on magnetic stirrer.
3. Add one-tenth volume of carbonate–bicarbonate buffer.
4. Add one-tenth volume of FITC solution dropwise while stirring the immunoglobulin solution at 4° (approximately 1 mg of dye per 100 mg of protein).
5. Check pH after addition of FITC and if necessary adjust to pH 9.0 with 0.1 *N* NaOH.
6. Cover reaction vessel and stir *gently* at 4° overnight. (Alternatively the reaction can be carried out at room temperature for 1–2 hr if the volume to be labeled is less than 20 ml.)

Removal of unreacted free FITC is preferably performed by dialysis followed by gel filtration chromatography on Sephadex G-50 (medium).

7. Dialyze conjugate against several changes of phosphate-buffered saline (PBS).
8. Prepare Sephadex G-50 column equilibrated with PBS such that the packed volume is at least six times the volume of conjugate to be applied. Allow a disc of filter paper, cut to fit the dimensions of the column, to float onto the top of the column. This facilitates the even application of conjugate.
9. Allow the PBS to run through the column until no buffer remains above the top of the column.
10. Stop the flow of buffer and apply the conjugate.
11. Allow the conjugate to flow into the column by opening the tap. When all the conjugate has passed into the column elute with PBS.
12. Collect the first colored peak to emerge (this contains the labeled immunoglobulins) and concentrate to the original conjugate volume.
13. Conjugates can be stored at 4° or in aliquots at − 20° after the addition of a preservative such as 0.1% sodium azide. Repeated freezing and thawing is to be avoided.

Peroxidase Labeling of Antibody Globulins

Although a variety of methods can be used for coupling enzymes to antibody,[28] the conjugation procedures most commonly used with horse-

[28] S. Avrameas, T. Ternynck, and J. L. Guesdon, *Scand. J. Immunol.* **8**, *Suppl.* **7**, 7 (1978).

radish peroxidase (HRP) are the two-stage glutaraldehyde[29] and periodate oxidation[30] methods. In the former procedure peroxidase is first mixed with an excess of the dialdehyde glutaraldehyde, which reacts with free amino groups of the enzyme via only one of its active aldehyde groups. After gel filtration chromatography to remove excess glutaraldehyde, the activated enzyme is mixed with the immunoglobulin preparation to allow the free aldehyde group to combine with an amino group of the immunoglobulin. Conjugates prepared in this way have been shown to contain a homogeneous derivative[29,31] with a molecular weight of 90,000, but the coupling efficiency is poor at around 25% and 5% for antibody and enzyme, respectively.[32] The low efficiency in this system appears to be due to the relative paucity of reactive amino groups in HRP. In contrast the periodate oxidation method of conjugation[30,33] is not dependent on the presence of reactive amino groups but relies upon the generation of active aldehyde groups after periodate oxidation of the carbohydrate moiety of peroxidase. These aldehyde groups combine with the amino groups of added immunoglobulin to form Schiff bases, which are subsequently stabilized by reduction with sodium borohydride. Conjugates prepared by this procedure contain high molecular weight derivatives,[30,32] but the coupling efficiency is increased to approximately 60% for both antibody and enzyme.[34]

Recent studies using a modification of the method described by Kato *et al.*[35] have shown that peroxidase can be satisfactorily coupled to antibody by coupling via sulfhydryl groups introduced into both the immunoglobulin and enzyme structures.[36] Conjugates prepared in this way contain active derivatives that are heterogeneous in relation to molecular weight but retain good enzyme and antibody activity.[37]

Glutaraldehyde Conjugation Method[28]

Materials

Horseradish peroxidase RZ 3.0
Stock solution of glutaraldehyde, 25% in water

29 S. Avrameas and T. Ternynck, *Immunochemistry* **8**, 1175 (1971).
30 P. K. Nakane and A. Kawaoi, *J. Histochem. Cytochem.* **22**, 1084 (1974).
31 M. Mannick and W. Downey, *J. Immunol. Methods* **3**, 233 (1973).
32 D. M. Boorsma and J. G. Streefkerk, *J. Histochem. Cytochem.* **24**, 481 (1976).
33 M. B. Wilson and P. K. Nakane, *in* "Immunofluorescence and Related Staining Techniques" (W. Knapp, K. Holubar and G. Wick, eds.), p. 215. Elsevier/North-Holland, Amsterdam, 1978.
34 D. M. Boorsma, J. G. Streefkerk, and N. Kors, *J. Histochem. Cytochem.* **24**, 1017 (1976).
35 K. Kato, Y. Hamaguchi, H. Fukui, and E. Ishikawa, *J. Biochem.* **78**, 423 (1975).
36 P. D. Weston, J. A. Devries, and R. Wrigglesworth, *Biochim. Biophys. Acta* **612**, 40 (1980).
37 S. M. Chantler and L. S. Cooper, unpublished observations, 1978.

Phosphate buffer, 0.1 *M*, pH 6.8
Sephadex G-25
Isotonic saline
Immunoglobulin preparation, 5 mg/ml in saline
Carbonate–bicarbonate buffer, 0.5 *M*, pH 9.5
Lysine solution, 1.0 *M* pH 7
Phosphate-buffered saline, pH 7.5 (PBS)
Saturated ammonium sulfate
Glycerol

Procedure

1. Dissolve 10 mg of peroxidase in 0.2 ml of a freshly prepared 1:25 dilution of the stock glutaraldehyde solution in phosphate buffer and allow to stand at room temperature for 18 hr.
2. Pass through Sephadex G-25 column equilibrated with saline to remove excess glutaraldehyde.
3. Collect the brown fractions, which contain the activated peroxidase, pool, and concentrate to 1 ml.
4. Add 1 ml of immunoglobulin solution (previously dialyzed against saline) to the peroxidase solution.
5. Add 0.2 ml of carbonate–bicarbonate buffer and leave for 24 hr at 4°.
6. Add 0.1 ml of lysine solution and leave the mixture at 4° for 2 hr.
7. Dialyze against several changes of PBS at 4°. If desired remove free enzyme by precipitation with saturated ammonium sulfate as described in steps 8–10.
8. Add an equal volume of saturated ammonium sulfate to the conjugate and allow to stand at 4° for 30 min.
9. Centrifuge for 20 min at 4000 *g* and discard supernatant.
10. Dissolve precipitate in approximately 1 ml of saline and dialyze extensively against several changes of PBS. (Alternatively, sulfate ions may be removed by gel filtration chromatography on Sephadex G-50.)
11. Preserve by adding an equal volume of glycerol, and store at 4°.

Periodate Oxidation Conjugation Method

Two procedures have been described by Nakane and co-workers. In the first of these[30] free amino groups on the peroxidase are blocked by fluorodinitrobenzene (FDNB) treatment prior to the production of active aldehyde groups by periodate oxidation. A recent modification of this method, described here, omits FDNB blocking and recommends periodate oxidation of the enzyme at low pH prior to coupling with immunoglobulin.[33]

Materials

Horseradish peroxidase RZ 3.0 (HRP)
Sodium metaperiodate (freshly prepared), 0.1 *M*
Acetate buffer, 1 m*M*, pH 4.4
Carbonate–bicarbonate buffer, 10 m*M*, pH 9.5
Immunoglobulin preparation
Carbonate–bicarbonate buffer, 0.2 *M*, pH 9.5
Sodium borohydride
Sephacryl S-200
Phosphate-buffered saline pH 7.5 (PBS)

Procedure

1. Dissolve 4 mg of HRP in 1 ml of distilled water.
2. Add 0.2 ml of freshly prepared periodate to the enzyme solution and stir for 20 min at room temperature.
3. Dialyze against acetate buffer overnight at 4°.
4. Prepare globulin solution containing 8 mg of protein in 1 ml of 10 m*M* carbonate–bicarbonate buffer.
5. Adjust activated HRP solution to approximately pH 9 by addition of 20 μl of 0.2 *M* carbonate–bicarbonate buffer.
6. Immediately add the globulin preparation to the HRP–aldehyde and stir for 2 hr at room temperature.
7. Add 0.1 ml of freshly prepared sodium borohydride solution containing 4 mg/ml and leave at 4° for 2 hr.
8. Separate unreacted enzyme from the mixture by chromatography on a column of Sephacryl S-200 equilibrated with PBS or by salt precipitation with ammonium sulfate as described above.
9. If purification of conjugates is performed by gel chromatography, the appropriate fractions should be pooled and concentrated prior to storage at − 20°. Addition of albumin (10 mg per milliliter of conjugate) or an equal volume of glycerol prior to freezing in small aliquots is recommended. Repeated freezing and thawing should be avoided.

Evaluation of Conjugates

A variety of tests should be used to determine the efficiency of conjugation and the suitability of the conjugate in use. The extent of the testing performed, particularly with respect to specificity, will vary with the intended use of the reagent.

Efficacy of labeling can be determined very simply by measuring the absorbance of the conjugate both at the 280 nm protein peak and at the maximum absorbance wavelength of the label used. For immunohistological studies the ratio of OD_{495} to OD_{280} for fluorescein-labeled reagents should lie between 0.6 and 0.9; and the ratio of OD_{403} to OD_{280} for peroxidase-labeled conjugates, between 0.3 and 0.6. This test, however, fails to show whether biological activity is present in the conjugate. This should be determined initially by using the conjugate as the antibody in appropriate gel diffusion or immunoelectrophoresis tests (if a suitable soluble antigen preparation is available), followed by testing in the immunofluorescent or immunoenzyme system in which it is to be used. Performance testing by titration (direct method) or chessboard titration (indirect method) is *essential* in order to select the optimal working dilution of the reagent and to assess its specificity under working conditions. Tests of immunological specificity carried out by other methods (e.g., gel diffusion) are irrelevant and may even give misleading results because of the widely varying sensitivity shown by different test systems.[38]

Antibody Production by Lymphocyte Hybridomas[38a]

Conventional immunization by injection of antigen into an animal stimulates the production of a heterogeneous population of antibodies that differ in respect of both their affinity and their specificity. Although the immunization procedure or prior treatment of the recipient may be manipulated to favor the production of antibodies of predominantly high or low affinity, the specificity of the antibody response is less amenable to control and antibodies directed against each of several antigenic determinants present in the immunogen will usually be present. The extent of this heterogeneity of response will differ not only among members of different species but also in individual animals of the same species despite the use of identical immunogen preparations and immunization schedules. These biological factors influence both the ease and reproducibility with which antisera of the desired immunological specificity can be prepared. The application of cell fusion techniques for *in vitro* production of antibodies of defined specificity offers a significant potential alternative to conventional methods of reagent antibody production.

In 1973, Cotton *et al.*[39] successfully fused cells of two plasmacytoma lines to produce hybrid cells capable of synthesizing both myeloma proteins. Subsequently, hybrid cells derived by fusion of a murine myeloma with spleen cells from appropriately immunized donors were shown to se-

[38] S. M. Chantler and M. Haire, *Immunology* **23**, 7 (1972).

[38a] The authors wish to acknowledge the helpful criticism of Dr. Jane Hewitt during the preparation of this section.

[39] R. G. H. Cotton, D. S. Secherand, and C. Milstein, *Eur. J. Immunol.* **3**, 135 (1973).

crete antibodies against the immunogen used.[40] These hybrid cells (hybridomas) could be grown in tissue culture, producing antibodies of defined specificity *in vitro*; alternatively, antibody secretion could be obtained *in vivo* by inoculation of the hybridoma cells subcutaneously or intraperitoneally into syngeneic recipients. This approach thus offered the possibility of the production of monoclonal antibody of defined specificity by selective cloning procedures, avoiding the need for highly purified immunogen or elaborate antibody purification procedures.

Although it is now well established that the fusion of mouse myeloma cells and antibody-secreting splenic lymphocytes is an effective means of producing homogeneous antibody of defined specificity, a number of technical variables remain. The same basic principles are applicable to many systems, but fairly extensive preliminary investigation is required to define the optimal conditions, particularly in relation to the choice of immunization schedule and donor species used. Investigators interested in detailed methodology should refer to the recent proceedings of a workshop on lymphocyte hybridomas.[41]

Choice of Fusion Partners

The myeloma line selected should exhibit good growth characteristics *in vitro*, a high fusion frequency (one hybrid per 10^5 to 10^6 normal cells) and should be sensitive to the selective medium HAT. If the cell line is lacking in either of the enzymes hypoxanthine guanine phosphoribosyltransferase (HGPRT) or thymidine kinase (TK), growth in this selective medium (which contains hypoxanthine, aminopterin, and thymidine) will be impossible.[42] Only after hybridization with a normal cell containing the enzymes can DNA synthesis and growth occur; thus hybrid cells alone survive in the selective medium. A limited number of myelomas exhibiting these features are available, and the one most commonly used is P3-X63Ag8 of BALB/c origin.

Hybrids obtained by fusion of an antibody-secreting normal cell and a myeloma cell such as the above produce specific antibody together with the myeloma protein and the products of mixed genetic combinations. This heterogeneous immunoglobulin production may not always pose a problem, but in applications where greater purity is necessary the difficulty can be avoided by using a nonsecreting myeloma line that produces

[40] G. Köhler and C. Milstein, *Nature (London)* **256**, 495 (1975).
[41] F. Melchers, M. Potter, and N. L. Warner, eds., "Lymphokine Hybridomas," *in Curr. Top. Microbiol. Immunol.* **81**, (1978).
[42] J. W. Littlefield, *Science* **145**, 709 (1964).

no immunoglobulin of its own but still supports the synthesis of spleen cell-derived immunoglobulins.[43,44]

The phylogenetic relationship between the cells utilized in hybridization studies determines the functional success of the hybrids produced. Murine myeloma lines have been successfully fused to both syngeneic and allogeneic mouse spleen cells[45,46] and to rat spleen cells,[47] but fusion with human lymphocytes and with cells of rabbit or frog origin has been less successful.[48] Recently Galfre and his colleagues[49] have described a rat myeloma line that has been successfully fused to rat spleen cells, but as yet no suitable human myeloma lines are available. The ontological derivation of potential fusion partners is also important.[50] It appears that optimal results are dependent upon fusion with cells of the B lymphocyte series at an appropriate stage of differentiation. Although the exact characteristics of the cell have not been identified, an activated B lymphocyte at an early stage of differentiation appears to be preferable. It follows therefore that selection of fusion partners of compatible phylogeny and ontogeny together with preselection of suitably differentiated B lymphocytes will increase the success rate of obtaining functional hybridomas. In practice, splenic cells from immunized mice have been most extensively used in experimental work because of the availability of suitable murine myelomas.

Although the myeloma line (P3-X63Ag8) commonly used in fusion studies is derived from the BALB/c mouse strain, it is not essential to use this inbred strain as a source of donor cells. Instead, it is preferable to use a strain that provides the best response to the immunogen in question; however, if it is intended finally to inoculate the hybrid clones into animals in order to produce antibodies *in vivo*, then clearly the recipient animal must be histocompatible. This can be achieved by using, as recipients, F_1 hybrids of BALB/c and the strain selected for initial immunization.

[43] M. Schulman, C. D. Wilde, and G. Köhler, *Nature (London)* **276**, 269 (1978).
[44] G. Köhler, S. C. Howe, and C. Milstein, *Eur. J. Immunol.* **6**, 292 (1976).
[45] G. Köhler and C. Milstein, *Eur. J. Immunol.* **6**, 511 (1976).
[46] G. Köhler, T. Pearson, and C. Milstein, *Somatic Cell Genet.* **3**, 303 (1977).
[47] G. Galfre, S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard, *Nature (London)* **266**, 550 (1977).
[48] G. Köhler and M. J. Schulman, *in* "Lymphocyte Hybridomas" (F. Melchers, M. Potter and N. L. Warner, eds.), *Curr. Top. Microbiol. Immunol.* **81**, 143 (1978).
[49] G. Galfre, C. Milstein, and B. Wright, *Nature (London)* **277**, 131 (1979).
[50] P. Coffino, B. Knowles, S. Nathenson, and M. D. Scharff, *Nature (London), New Biol.* **231**, 87 (1971).

Immunization Procedure

In most somatic cell hybridization studies the potential spleen cell donor is immunized in order to increase the proportion of cells producing specific antibody. This enrichment of functionally active cells has been shown to increase the percentage of hybridomas exhibiting the desired specific antibody activity. The type of immunization schedule adopted will depend upon the physical nature of the antigen and its immunogenicity, so that the variables, such as use of adjuvant, route of injection, and the timing of injections, will differ in different studies. Immunization commonly involves an initial subcutaneous injection of immunogen followed by a booster intravenous injection. The animals are tested 2–5 days after the boost, and a good responder is given a second intravenous injection, spleen cells being harvested 2–5 days later.

Preparation of Spleen Cells

Separation of nucleated cells from red blood cells present in the spleen cell suspension is rarely performed. Spleen cells are washed twice in serum-free medium, the yield from one spleen being approximately 1×10^8 nucleated cells. A ratio of 10 spleen cells : 1 myeloma cell is used for fusion. If it is possible to enrich the proportion of plaque-forming cells in the spleen suspension—for instance, by rosetting with antigen-labeled red blood cells followed by centrifugation in Ficoll-Isopaque—the ratio used for fusion may be reduced to 1 : 1. Such an enrichment procedure not only decreases the number of cells that need to be distributed into individual culture wells after fusion, but also increases the percentage of hybridomas that secrete antibody of the desired specificity, thereby reducing the number of tests performed in the selection of appropriate hybrid clones at a later stage.

Cell Fusion

In early studies fusion was promoted by the use of Sendai virus, but more recently polyethylene glycol (PEG) of molecular weight 1000–6000 has been preferred. The sediment of spleen and myeloma cells is gently resuspended in the small volume of washing medium remaining after centrifugation, and approximately 2 ml of 50% PEG solution diluted in the serum-free medium is added. After incubation at 37° for 1 min, the cell mixture is diluted *slowly* with medium, approximately 5 ml being added over a period of 5 min. The suspension is then centrifuged and resuspended in the selective HAT medium (containing serum) to a final density of approximately 10^6 cells per milliliter. This procedure yields approximately 100 ml of suspension from one spleen.

Growth of Hybrid Cells

The fused cells, suspended in HAT medium, are seeded into individual tissue culture wells, putting approximately 10^6 cells into each well. Unfused myeloma cells cannot grow in this selective medium, and normal spleen cells are incapable of prolonged growth, so only the hybrid cells survive. These "microcultures" are examined periodically, and those showing growth visible over approximately 30% of the base of the individual wells are tested for specific antibody activity, this stage being reached in successful wells between 7 and 20 days after seeding. The percentage of wells showing growth will depend on the number of cells originally introduced: approximately 90% of wells exhibit growth when 10^6 cells are placed in each culture well. The majority of the wells will contain multiple clones derived from different parent hybrid cells, the products of many of which are irrelevant to the particular study. The proportion of wells containing functional hybrids of the desired specificity will vary considerably, but approximately 5% of those showing growth may contain appropriate hybrids.

Evaluation of Activity of Hybrid Products

The supernatants obtained from individual culture wells exhibiting growth must be tested to determine whether any hybrids present in that culture are secreting antibody of the required specificity. Since the level of immunoglobulin secretion is low (approximately 10–50 $\mu g/ml$) and the number of wells to be tested may be relatively large, it is essential that highly sensitive and specific assays that are readily performed on small volumes of supernatants be used for screening. Radioimmunoassays are most widely used, but hemagglutination, hemagglutination inhibition, and (in cases where localization of activity is relevant) immunofluorescence and immunoenzyme procedures have been applied.

Cloning of Active Hybrids

As previously mentioned, culture wells containing antibody of the appropriate specificity may contain a heterogeneous population of hybrid cells secreting a variety of products. Individual hybrid cells can be separated only by additional cloning procedures, either by growth in soft agar or by using the limiting dilution method.

Cloning by the soft agar method is carried out in petri dishes 3 cm in diameter that contain a layer of normal spleen "feeder" cells (10^6 per plate) in 5% agar, over which is then layered a dilution of the hybrid cells (obtained from positive wells) suspended in a medium containing 20% fetal calf serum in 2.5% agar. A range of different dilutions of the hybrid

cells may be treated in this way. After incubation, individual clones of cells are detectable within 1–2 weeks. These discrete colonies are then transferred to microculture wells and their products are again tested for activity of the required specificity. Cultures exhibiting appropriate activity are immediately recloned at least twice in order to select stable functional cell lines, which are stored by freezing in vials or transferred to larger culture vessels.

The limiting dilution method of cloning involves culturing serially diluted suspensions of hybrid cells together with normal spleen cells, each dilution being set up in 6–12 wells. The average number of hybrid cells dispensed in each series lies between 240 and 0.1 cells per well. At high cell levels growth is observed in most wells, but statistical considerations suggest that if only one-third of the wells seeded at a particular cell dilution show growth, then it is highly probable that the cells growing within each of the individual wells are derived from a single parent cell. These wells are then tested for antibody activity, and the cellular contents are recloned to establish functional stability in the same way as those derived by soft agar cloning procedures.

Antibody Production

Once stable hybrid clones secreting antibody of defined specificity have been isolated, methods of obtaining maximal amounts of antibody become important. These may involve *in vitro* culture or *in vivo* growth in a suitable recipient. The hybridomas may be maintained in continuous culture *in vitro* for several months at a cell density within the range of 10^4 to 4×10^6 cells per milliliter. Under these conditions an antibody yield of 10–100 μg/ml can be obtained, but in most cases loss of functional activity eventually occurs. The reason for such functional instability is not clear, but it is likely to be due to loss of chromosomes during a period of time in culture. For this reason *in vitro* antibody production is more satisfactorily performed in limited rather than continuous culture, selected stable clones being stored by freezing at an early stage in their life cycle so that a new vial of cells can be thawed when required to initiate a fresh culture.

As an alternative, antibody can be produced *in vivo*. Many cultured hybridomas have been successfully transplanted to genetically compatible recipients,[45,49,51] and hybridomas derived from cells of nonmurine origin have been successfully transplanted to athymic nude mice.[52] *In vivo* antibody production is achieved by inoculating the cloned, hybrid cells

[51] T. Pearson, G. Galfre, A. Ziegler, and C. Milstein, *Eur. J. Immunol.* **7**, 684 (1977).

[52] H. Koprowski, Z. Steplewski, D. Herlyn, and M. Herlyn, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3405 (1978).

subcutaneously or into the peritoneal cavity. If the latter route is used, mineral oil is given several days prior to inoculation in order to encourage the production of ascitic fluid. The level of antibody obtained by *in vivo* culture is reported to be 100- to 1000-fold greater than for *in vitro* culture.[39,45,49,51]

In assessing the efficacy of *in vitro* versus *in vivo* production of reagent antibody, consideration must be given both to the relative concentrations of antibody and to the volumes obtainable. Subcutaneous inoculation of cells in a mouse yields approximately 1 ml of serum 2 weeks later; the yield of ascitic fluid harvested 7–14 days after intraperitoneal injection is between 5 and 15 ml. As the concentration of antibody produced in this way is at least 100-fold greater than in tissue culture, 10 ml of ascitic fluid from one mouse would be equivalent to at least 1 liter of tissue culture fluid. In this context, the recent description of hybridomas produced by fusion of a rat myeloma line with rat spleen cells is likely to be of considerable practical significance because of the larger volume of serum obtainable following inoculation of hybridomas in these rodents.[49]

Nonhybridoma Techniques

The production of a thriving, functional hybridoma is dependent on a close phylogenetic relationship between the two parent cell lines; the only species to have provided suitable cell lines so far are mice and rats. An alternative approach to the production of nonrodent antibodies in cell culture is provided by the transformation of B lymphocytes on exposure to Epstein–Barr virus (EBV). Adult human peripheral blood cells exposed to EBV have been shown to release polyclonal secretory immunoglobulin.[53] Cultures of human peripheral blood lymphocytes exposed to antigen (sheep red blood cells) and EBV produce specific antibody.[54] Preselection of human peripheral blood lymphocytes exhibiting surface binding of tetanus toxoid and the hapten NNP (4-hydroxy-3,5-dinitrophenacetic acid) followed by viral transformation has been shown to yield cells capable of antibody production *in vitro*.[55,56] Although the cultures have been shown to be active for some months, methods of increasing both the yield of antibody (at present only some 10 ng per milliliter of culture fluid) and long-term stability have yet to be devised.[57] Attempts to establish stable spe-

[53] A. Rosen, P. Gergely, M. Jondal, and G. Klein, *Nature (London)* **267**, 52 (1977).

[54] A. L. Luzzatti, H. Hengartner, and M. H. Schreier, *Nature (London)* **269**, 419 (1977).

[55] V. R. Zurawski, E. Haber, and P. H. Black, *Science* **199**, 1439 (1978).

[56] M. Steinitz, G. Klein, S. Koskimies, and O. Makel, *Nature (London)* **269**, 420 (1977).

[57] V. R. Zurawski, S. E. Spedden, P. H. Black, and E. Haber, *in* "Lymphocyte Hybridomas" (F. Melchers, M. Potter, and N. L. Warner, eds.), *Curr. Top. Microbiol. Immunol.* **81**, 152 (1978).

cific antibody-secreting cell lines by somatic hydridization with the murine myeloma P3-X63Ag8 have been unsuccessful.[58]

Summary

The successful fusion of normal and neoplastic lymphocytes has laid the foundation for the production of a variety of antibody specificities of practical relevance in research and diagnosis. The technical problems associated with this approach should not be underestimated, but one cannot fail to recognize the enormous range of applications that lie ahead once these problems have been overcome.

[58] H. Hengartner, A. L. Luzzatti, and M. Schreir, *in* "Lymphocyte Hybridomas" (F. Melchers, M. Potter, and N. L. Warner, eds.), *Curr. Top. Microbiol. Immunol.* **81**, 92 (1978).

[6] Preparation of Fab Fragments from IgGs of Different Animal Species

By MICHAEL G. MAGE

The light and heavy polypeptide chains of the IgG molecule are folded into a series of globular regions called domains[1] (Fig. 1). The portion of the polypeptide chain between the Cγ1 and Cγ2 domains of the heavy chain, known as the "hinge region,"[2] is relatively accessible to proteolytic enzymes. When whole IgG molecules are incubated with the proteolytic enzyme papain, in the presence of low concentrations of sulfhydryl compounds, one or more peptide bonds in the hinge region are split,[3] leading to the release of the Fab and Fc fragments (Fig. 1).

The Fab fragments of IgG antibodies thus consist of the light chain, and the V_H and Cγ1 domains[1] of the heavy chain. Fab fragments are univalent, in that each fragment contains a single antibody combining site, composed of parts of the variable regions (V_L and V_H) of the light and heavy chains. Because of their univalency, Fab fragments can be used to advantage in procedures where it is desirable to bind antigen to antibody in solution without cross-linking or precipitation or to bind to antigen on cell surfaces without producing "patching" or "capping."[4]

[1] G. M. Edelman and W. E. Gall, *Annu. Rev. Biochem.* **38**, 415 (1969).
[2] D. S. Smyth and S. Utsumi, *Nature (London)* **216**, 332 (1967).
[3] S. Zappacosta, A. Nisonoff, and W. J. Mandy, *J. Immunol.* **100**, 1268 (1968).
[4] F. Loor, L. Forni, and B. Pernis, *Eur. J. Immunol.* **2**, 203 (1972).

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